DEVELOPMENT TOWARDS A PROTOCOL TO TEST FOR THE CREATION OF A "DIABETIC" ENVIRONMENT IN A BLOOD VESSEL MIMIC

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Introduction

Overview

Diabetes mellitus affects a huge proportion of people; about 6% of the population in the US had diabetes in 2007 (16). Diabetes promotes dyslipidemia, an imbalance in the circulating levels of lipids and lipoproteins, and diabetes is one of the leading risk factors for coronary artery and peripheral vascular diseases (11, 51, 52). There are two types of diabetes; type 1 diabetes accounts for about 10% of diagnosed cases and type 2 accounts for about 90% (11). Diabetic patients react differently to stents and there is a great need to enhance the treatment. The goal of this project was, and continues to be, to create a "diabetic" environment in which stents can be tested to characterize the potential of the stent to continue with animal testing.

Cardiovascular Disease

Early in the progression of cardiovascular disease, endothelial dysfunction occurs. The progression of endothelial dysfunction begins when the endothelial cells are activated and express adhesion molecules such as intracellular adhesion molecule (ICAM). From here the leukocytes stick to the adhesion molecules and migrate into the vessel. The smooth muscle cells (SMCs) then migrate towards the intima and proliferate and the beginning of a lesion is formed (32). Once the circulating lipoproteins bind, there is a sustained inflammatory response and the lesion starts to grow and calcify; lipids begin to accumulate and soon a significant lesion is formed. This build-up of plaque is known as atherosclerosis and is illustrated in Figure 1.1. When this first occurs the vessel will try to compensate by remodeling and expanding so the lumen remains the same size. However, eventually the blood vessel narrows and blood flow is affected. There are two

main problems due to this progression. First, the narrowing of the vessel impedes the blood flow and the tissue doesn't receive the proper amount of oxygen and nutrients. Second, the lesion can rupture and create a clot.

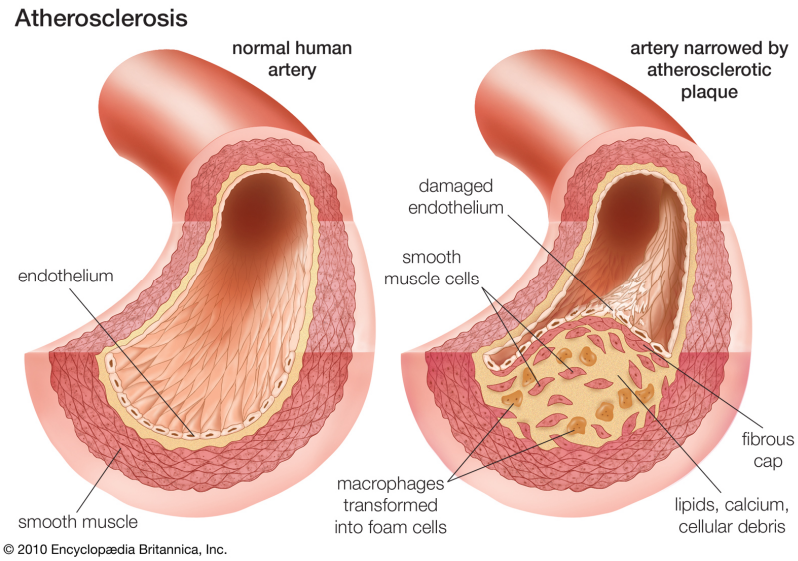

Figure 1.1- Progression of atherosclerosis in cardiovascular disease (1).

The current treatments for cardiovascular disease are lifestyle modifications, drugs, angioplasty and/or stents, and bypass surgery. In angioplasty a balloon is used to push the plaque against the vessel wall and often times a stent remains to prevent restenosis. Stents are a huge area of research and are widely used in the clinic (cite). Many new materials, such as biodegradable and drug eluting stents are currently being researched (48). Biodegradable stents will provide the support long enough to prevent restenosis, but degrade in time to allow for endothelialization- preventing late stent thrombosis. Drug eluting stents are used to prevent the proliferation of cells, or restenosis, but often times they prevent endothelialization and healing. Once a desired stent has been developed, it must go through the FDA prior to market approval in the U.S.

In order to obtain FDA approval to market, much testing is required. Kristen Cardinal's Tissue Engineering Laboratory at California Polytechnic State University has created an *in vitro* blood vessel mimic (BVM) to test stents and obtain data before the stent is tested in animals. The BVM consists of an 8-roller pump, a reservoir for media and a bioreactor for the engineered vessel. This set up can be seen in Figure 1.2. This is a very valuable tool to save time, money and resources. Stents can be tested in a more high throughput manner *in vitro*, and the stents that show the most potential can then be tested further in animal models.

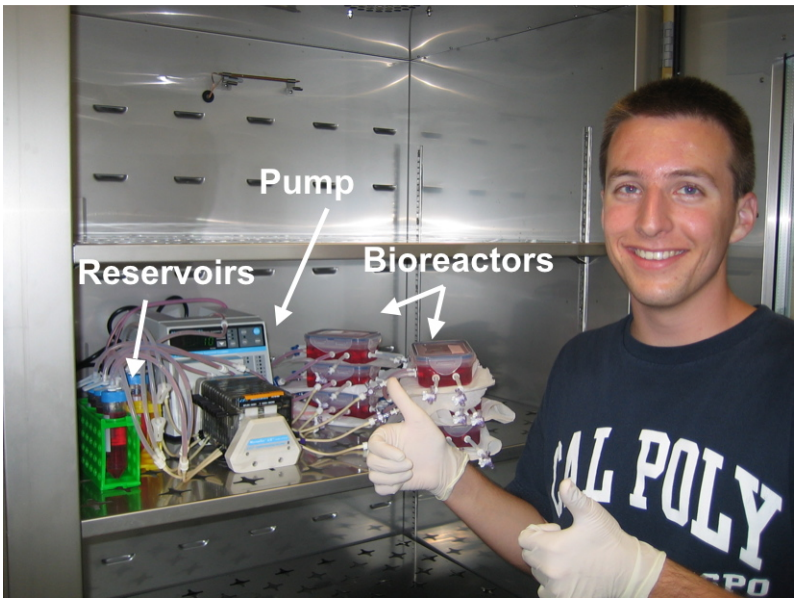

Figure 1.2- Blood vessel mimic as used to test stent characteristics.

Diabetes Mellitus

There are two different types of diabetes; type 1 diabetes and type 2 diabetes. Type 2 diabetes is often associated with obesity, lack of exercise, and unhealthy eating choices (11). In this type of diabetes, insulin sensitivity is decreased, which ultimately leads to inadequate release of insulin and β-cell failure. This can be apparent at either the insulin receptor level or postreceptor signaling (11, 25). There are a few leading causes of type 2 diabetes. The first is a decrease in the ability to increase the GLUT4 uptake of glucose. This could be due to an increase in triglycerides (TG) in the muscle due to an unhealthy diet causing hyperinsulemia (11). Initially, more glucose will be uptaken into the skeletal muscle and this will increase lipogenesis and repress fatty acyl CoA oxidation (11). Some of these byproducts, diacylglycerol and ceramide, will stimulate signaling pathways as they accumulate, which disrupts signaling from the insulin receptor (IR) and insulin receptor substrate (IRS) proteins (11). This will then create insulin resistance in the skeletal muscle (11). The second cause of obesity-induced insulin resistance is the decreased ability of insulin to repress hepatic glucose production (11). The liver creates glucose through glycogenolysis and gluconeogenesis (11). Insulin represses the enzymes in both of these pathways (11). The increase in visceral adipose tissue can induce the effects of lipotoxicity by releasing cytokine tumor necrosis factor-α (TNF-α), which can disrupt insulin signaling pathways (11). The third common reason for insulin resistance is the inability of insulin to repress hormone-sensitive lipase (HSL) or increase lipoprotein lipase (LPL) in adipose tissue (11). High HSL and low LPL are commonly found in people with insulin resistance (11). This dyslipidemia is characterized as hypertriglyceridemia with large TG-rich VDL particles that are produced by the liver (11). These particles are digested efficiently creating small dense LDL particles, known to be atherogenic (11). HDL, which protects against vascular disease,

has also been shown to take on TG instead of cholesterol, which shortens the half-life of HDL and decreases the HDL levels (11).

Type 1 diabetes is an autoimmune disease of the β-cells and is often onset in childhood. β-cells produce insulin, and in Type I diabetics beta cells are destroyed, so these patients are insulin dependent and need exogenous insulin to prevent ketosis. It is believed that the initial stimulus can be due to a viral infection, which recruits leukocytes to islets or dendritic cells in lymph that drain from islets and will present antigens to T-cells. This leads to insulitis, or inflammation of insulin producing cells, and ultimately β-cell destruction. This disease can also be due to cytotoxicity, and it has been found that NKp46, a receptor on CD8+ T-cells, is required for β-cell toxicity in animal models. The inappropriate T-cell activation can also be caused by a couple of factors. First, glutamic acid decarboxylase 65 (GAD65) is an enzyme found outside of cells; often times in type 1 diabetes the T-cells are reactive to GAD65. Second, the major histocompatibility complex (MHC) genes may cause inappropriate T-cell activation. Third, there can be an inappropriate expression of the insulin gene in the thymus, which is where the T-cells mature. All of these can lead to type 1 diabetes.

Endothelial Cell Biology and Dysfunction

In the use of an *in vitro* BVM it is important to understand the structure and function of the artery that is being mimicked. The blood vessel system can be subdivided into three different components: arterial system, venous system and microcirculation (11). There are a couple of key differences between each of these blood vessel systems.

The arterial system distributes blood from the heart to the tissue, the venous system delivers blood to the heart from the tissue, and the microcirculation system connects the arterial and venous systems and allows for waste and nutrient exchange (11). The capillaries of the microcirculation system consist of fenestrated and non-fenestrated endothelial cells (ECs) as well as continuous and discontinuous ECs (5). Both the venous and arterial systems are composed of only non-fenestrated, continuous endothelium (ECs are tightly connected and surrounded by a continuous basement membrane) and the arterial system also contains many tight junctions (5, 6). The ECs of the venous system are phenotypically shorter and wider than those of the arterial system. The pressure in the venous system is not as high as the arterial pressure and the venous system has valves to prevent the back flow of blood. The venous system also has thinner walls. For the reminder of this report, the arterial system will be the primary focus because the problem lies in getting blood out to the body.

The artery is made of three layers: adventitia, media and intima, as illustrated in Figure 1.3. The adventitia, the outermost layer, is composed of collagen, fibroblasts, nerves (in the larger arteries) and vasovorum. The middle layer, the media, is composed of smooth muscle cells (SMC) and elastin. The SMCs align perpendicular to blood flow and therefore, wrap around the vessel. The inner layer, the intima, is composed of a basement membrane and endothelium, which is composed of ECs. The ECs within the artery align parallel to flow and are supported by the basement membrane. The ECs are the blood contacting surface and tell the smooth muscle cells (SMCs) when to constrict (4). The major function of the artery is to act as a conduit for blood, to provide an anti-thrombogenic lining, control blood cell trafficking, control permeability and help control flow through dilation and constriction (4, 5).

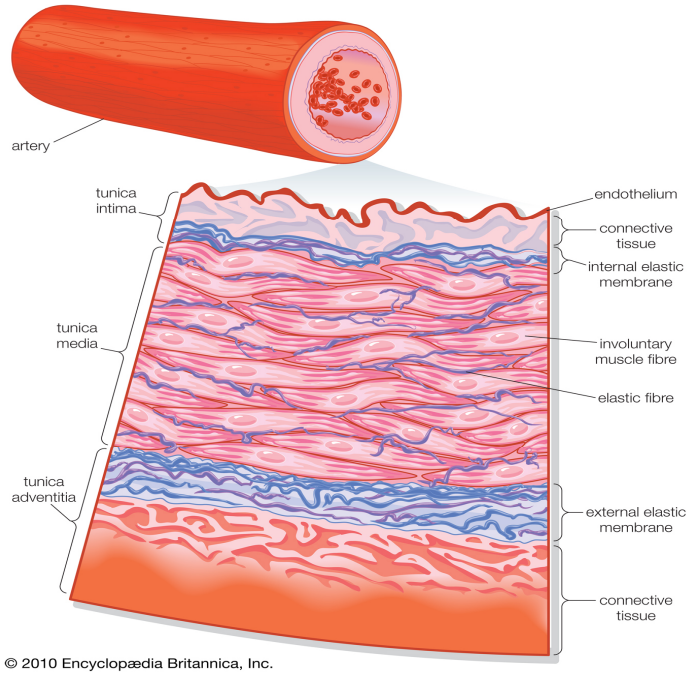

Figure 1.3 – The three arterial layers- adventitia, media and intima (1).

When using ECs in the laboratory or in tissue engineering, it is important to note their source. ECs from capillaries in the skin, liver and brain all have very distinctive properties and different surface protein expressions (15). The control of the plasticity of the artery- and vein-specific properties is not completely understood. There are studies supporting the hypothesis that these properties are epigenetically programmed before blood flow. For example, the stimulation of arterial ECs with oxidized LDL had much greater effects on the adhesion, proliferation and apoptosis than ECs from a human saphenous vein (5). However, as in coronary artery bypass grafting, when veins are grafted into the arterial system their walls thicken, permeability decreases, endothelial nitric oxide synthase (eNOS) increases and thrombomodulin decreases (6). This lends support to the hypothesis that structure and function are controlled by the microenvironment. It is likely that the EC phenotype is controlled by both the microenvironment and epigenetics. Input from the microenvironment can be attributed to

shear stress, growth factors, cytokines, chemokines, hormones, nitric oxide, oxygen and reactive oxygen species (5). The phenotype of ECs can be determined or measured through cell shape, protein expression, migration, proliferation, apoptosis, vasomotor tone, hemostatic balance, release of inflammatory mediators, and leukocyte adhesion (5).

Endothelial cells are very important in the regulation of blood flow. This is controlled by initiating/modulating an inflammatory response, activating leukocyte trafficking, vasomotion and coagulation (33, 36). ECs express receptors for mediators on their surface and they can release biologically active mediators such as cytokines and hematopoietic growth factors (36). They produce and release nitric oxide (NO) and prostacyclin (PGI_2) both known vasodilators; as well as vasoconstrictors such as endothelin, platelet –activating factor (PAF), and angiotensin II (13, 15, 25). These compounds are regulated at the gene level; they are not present in the cell until a receptor causes gene transcription (15). The endothelium in arteries is not highly permeable due to the tight junctions (5). The endothelium can induce permeability when inflamed by the formation of intracellular gaps. The formation of gaps can be enhanced by histamine, serotonin, bradykinin and VEGF (5).

Leukocyte trafficking is accomplished through a multi-step process including attachment, rolling arrest and transmigration (5). After the initial attachment, the leukocyte interacts with endothelial E- and P-selectin to mediate the rolling (5). Next the leukocyte integrins interact with ICAM-1 and vascular cell adhesion molecule (VCAM)-1 (5). Finally, transmigration is mediated through the use of CD99, PECAM-1 (CD31) and junctional adhesion molecule-1 (5). E- and P-selectin are not expressed in quiescent ECs (5). ICAM-1 and VCAM-1 are expressed in areas that are predisposed to

atherosclerosis, however they can be induced by activation agonists such as lipopolysaccharide or tumor necrosis factor (TNF)-α (5). A study on mice showed that TNF-α can upregulate ICAM-1, VCAM-1, E-selectin and P-selectin (5). The increase in cell-adhesion molecules was correlated with increased adhesion of leukocytes (5).

Vasomotion is imperative in vessels. Healthy ECs release NO. NO diffuses to the SMCs to control vasomotion. NO is also active in the inhibition of platelet activation, prevention of SMC proliferation, monocyte adhesion, platelet aggregation and EC apoptosis. NO release is regulated by physical activity, shear stress and hormones. PGI_2 is also found in the endothelium and helps to inhibit platelet aggregation and prevent proliferation of vascular smooth muscle cells (13). Endothelium-derived hyperpolarizing factor (EDHF) is another important vasodilator; even when other factors are inhibited, this can persist. Endothelin (ET)-1, produced by ECs, causes vasodilation at lower concentrations and vasocontraction at higher concentrations (13). ET-1 enhances the effects of other vasoconstrictors such as serotonin and norepinephrine. After metabolization of ET, NO is helpful to restore the normal vasotone (13). Acetylcholine, histamine and serotonin activate a receptor on SMCs and platetlets to counteract the effects of NO and PGI_2 (13). Angiotensin II is also important for vasoconstriction. It helps maintain vascular tone, tissue formation and structure. Angiotensin-converting enzyme (ACE), which converts angiotensin I to angiotensin II, is involved in the breakdown of the stimulus bradykinin, needed for NO and PGI_2 release (13).

ECs are imperative to maintain blood in a fluid state, they protect the circulating blood from being exposed to prothrombotic subendothelial material (21). ECs release several important mediators to prevent platelet activation, adhesion and aggregation (21).

For anti-coagulation purposes, ECs in arteries express thrombomodulin, endothelial protein C receptor (EPCR), tissue-type plasminogen activator (t-PA), ecto-ADPase, prostacyclin, and NO (5). Vasodilators, PGI_2 and NO, help to prevent platelet adherence to the endothelium. NO has also been shown to limit the adhesion of monocytes, preventing the build up of atherosclerotic plaque. NO and PGI_2 are enhanced by thrombin, bradykinin, serotonin, PDGF and IL-1 (13). For procoagulation, ECs express tissue factor, plasminogen activator inhibitor (PAI)-1, von Willebrand factor (vWF), cellular adhesion molecules, and protease activated receptors (5). Angiotensin II up-regulates the expression of adhesion molecules causing circulating monocytes to adhere to the endothelial surface. It also leads to platelet aggregation, thrombosis and migration and growth of SMCs. In healthy ECs, angiotensin II is inhibited due to the presence of NO, so the vessel wall is normally in a state of quiescence.

The balance between damage and repair of the vessel is imperative for proper EC function (14). The damage process is not only dependent on the migration and proliferation of mature ECs, but also on endothelial progenitor cells (EPCs). There are three main cell types that contribute to vascular homeostasis. Endothelial colony forming cells (ECFC) are highly proliferative and originate in the bone marrow (47). ECFC can produce endothelial progeny, form endothelial tubes *in vitro* and contribute to neo-vascularization as well as re-endothelialization (47). The second type is circulating angiogenic cells (CAC). CACs originate from the monocyte-macrophage lineage and are involved in the uptake of acetylated low-density lipoprotein (LDL) (47). CACs produce angiogenic cytokines that contribute to vascular homeostasis (47). The third type is colony-forming units (CFU) composed of myeloid progenitor cells, monocytes and T

lymphocytes. CFUs are often used as a biomarker for cardiovascular disease as they have a high correlation with endothelial dysfunction.

There are many factors that can lead to systemic changes of the cardiovascular system including hypertension, hyperglycemia and hyperlipidemia. These cause disturbed flow, activation/dysfunction of ECs and ultimately inflammation, increased oxidative stress, reduced NO bioavailability and monocyte extravasation (6). Impaired NO production in conjunction with excessive oxidative stress causes a decline in NO bioavailability leading to EC apoptosis (47). EC dysfunction impairs the vasomotion and causes procoagulation, oxidative stress and inflammation and further progression to atherosclerosis and diabetic complications.

Endothelial Cell Dysfunction in Diabetics

There are a couple of pathways that lead to endothelial cell dysfunction and complications. The first is polyol-sorbitol; it leads to an increase in oxidant stress. The second, hexosamine leads to an increase in transcription of proinflammatory cytokines. The third, advanced glycation end product (AGE) decreases protease degradation of collagen, creating a stiffer vascular wall and increasing proinflammatory cytokine expression and PKC activation. AGE then binds with its cellular receptor leading to the release of growth factors and activation of inflammatory pathways (13). AGE begins due to chronic hyperglycemia and decreases NO delivery to SMCs. This can then lead to atherosclerosis. AGE has been found to accumulate more quickly than normal in patients with diabetes (51). The fourth pathway is direct activation of protein kinase C (PKC),

which leads to an increase in inflammation and oxidant stress. PKC is activated by an increase in diacylglycerol levels due to hyperglycemia. PKC then stimulates the NADPH oxidase enzyme leading to superoxide production and oxidative stress (13). In an effort to account for all of the different pathways involved in diabetes the following hypothesis has been developed: there is an increase of superoxide production in ECs, which activate the main pathways such as PKC and AGE to lead to EC dysfunction and atherosclerosis (13). This hypothesis implies that EC dysfunction in diabetic patients is complex and begins with an increase in oxygen free radicals and a decrease in NO bioavailability. This is followed by inflammation and eventually atherosclerosis.

EC dysfunction is characterized by a decrease in vasodilation. In diabetic patients, it has been found that there is a decrease in the formation of the vasodilator, PGI_2 (13). This occurs before any changes in the vessel structure, but can increase blood pressure. NO is also decreased in active ECs. Oxygen-derived free radicals lead to an increase in the degradation of NO by NADPH oxidase (13). Diabetes also leads to the impairment of EDHF-dependent vasodilation (13). ET-1 is often found to be increased in diabetic patients. It has been shown that ET-1 levels correspond to the number of vascular complications in diabetic patients with clinical angiopathy (13).

EC dysfunction in diabetic patients is characterized by procoagulation; platelets adhere to the ECs and aggregate. Most coronary events that occur are due to less than 1/3 narrowing of the vessel. ECs in regions of arteries with disturbed flow are primed for activation by having increased levels of nuclear factor κB (NF-κB) in their cytoplasm that can be translocated to the nucleus to increase the expression of procoagulants (5). AGE's receptor RAGE, can be activated by NF-κB leading to the expression of VCAM

and ICAM (51). Diabetic patients express a number of coagulation factors that can lead to increased levels of fibrinogen and thrombosis (13). There often is a defective response to PGI_2 and NO. With EC dysfunction, when platelets adhere to the vascular wall they cause vasocontraction, and proliferation and migration of SMCs. The decrease in NO along with the increase in various growth factors leads to proliferation.

EC dysfunction is also characterized by an increase in oxidative stress. This is caused by the production of ROS as well as AGE/RAGE. The reaction of AGE and RAGE can lead to a positive feed-back loop. AGE and RAGE induce the production of reactive oxygen species (ROS), which leads to NF-κB and further production of ROS and then more AGE products. This leads an the increase in O_2 and oxidative stress in diabetic vessels as well as a decrease in the bioavailability of NO (51).

EC dysfunction is associated with inflammation as well as atherosclerosis. EC dysfunction can be used as a predictor of cardiovascular events (51). Activated ECs increase leukocyte trafficking. Activated ECs release cytokines, this leads to the accumulation of T cells and foam cells that perpetuate inflammation, lipid accumulation and SMC activity. Some studies have shown that EPC numbers and function in diabetic patients with cardiovascular risk factors are decreased (21). These EPCs are imperative for endothelial repair and function. In summary, EC dysfunction is characterized by procoagulation, an increase in oxidative stress, increase in oxidative stress, increase in atherosclerosis and a decrease in vasodilation.

Implications of Endothelial Cell Dysfunction in Diabetic Patients

EC dysfunction is not always associated with diabetes. EC dysfunction is associated with atherosclerosis and cardiovascular disease, but one does not have to be diabetic to express these cardiovascular risk factors (21). There are many conditions predisposing of atherosclerosis and these include dyslipidemia, hypertension and diabetes. Diabetes, therefore, is a risk factor for atherosclerosis and EC dysfunction (33). Also, diabetic patients often have dyslipidemia and hypertension, both risk factors for atherosclerosis. Therefore, a diabetic patient is at much greater risk of developing atheroscerosis than a healthy individual because they often times have more risk factors. Diabetic patients also have high glucose levels if they are not able to carefully control their blood sugar levels (13). This increase in glucose level increases the superoxide generation and disturbs proliferation; endogenous antioxidant enzymes are then over-expressed and there is an increase in free radicals and a decrease in NO (13). A decrease in NO and increase in free radicals are both associated with EC dysfunction. Diabetes and EC dysfunction are closely tied together, however, they are not mutually exclusive. For the purpose of this project, EC dysfunction in diabetic patients will be the main focus.

There are many complications associated with diabetes and EC dysfunction. One complication is retinopathies, a major cause of blindness. There are non-proliferative as well as proliferative retinopathies. In non-proliferative retinopathy microaneurisms occur in the capillaries and block blood supply in the retina. As this becomes more severe, proliferative retinopathy develops. Proliferative retinopathy is characterized by the growth of new vessels in the retina. These vessels are very fragile and can leak blood causing vision blindness or vision loss. Often times the pressure can be relieved before causing blindness (3). Diabetic retinopathy is caused by a high intracellular glucose

concentration in retinal ECs and pericytes due to hyperglycemia causing endothelial cell dysfunction (11). Diabetes can cause peripheral nerve damage due to a diminished blood supply to nerves. Diabetes can also lead to reduced renal function, chronic ulcerations, and atherosclerosis.

Atherosclerosis is almost always present in diabetic patients after 5-10 years (13). In diabetic patients, atherosclerosis tends to develop earlier than in non-diabetic patients. Atherosclerosis can lead to myocardial infarction and stroke. LDL accumulates in the intima of the artery and activates the endothelial cells to express adhesion molecules. These molecules then adhere to monocytes and leukocytes, which migrate into the intima (24). The monocyte derived macrophages upregulate scavenger receptors (SR) and toll-like receptors (TLR), which initiate signaling cascades and release cytokines, proteases and vasoactive molecules leading to further inflammation and atherosclerotic build up. Pro-inflammatory cytokines, proteases and coagulation factors produced by macrophages as well as T cells can lead to degradation of the plaque and thrombosis. T cells in the lesion lead to a Th1 response that is involved with IFN-γ and TNF, both of which have been shown to promote atherosclerosis. The atherosclerotic progression can be seen in Figure 1.4 (24). Anti-inflammatory cytokines, regulatory T cells and protective antibodies can help to prevent atherosclerosis.

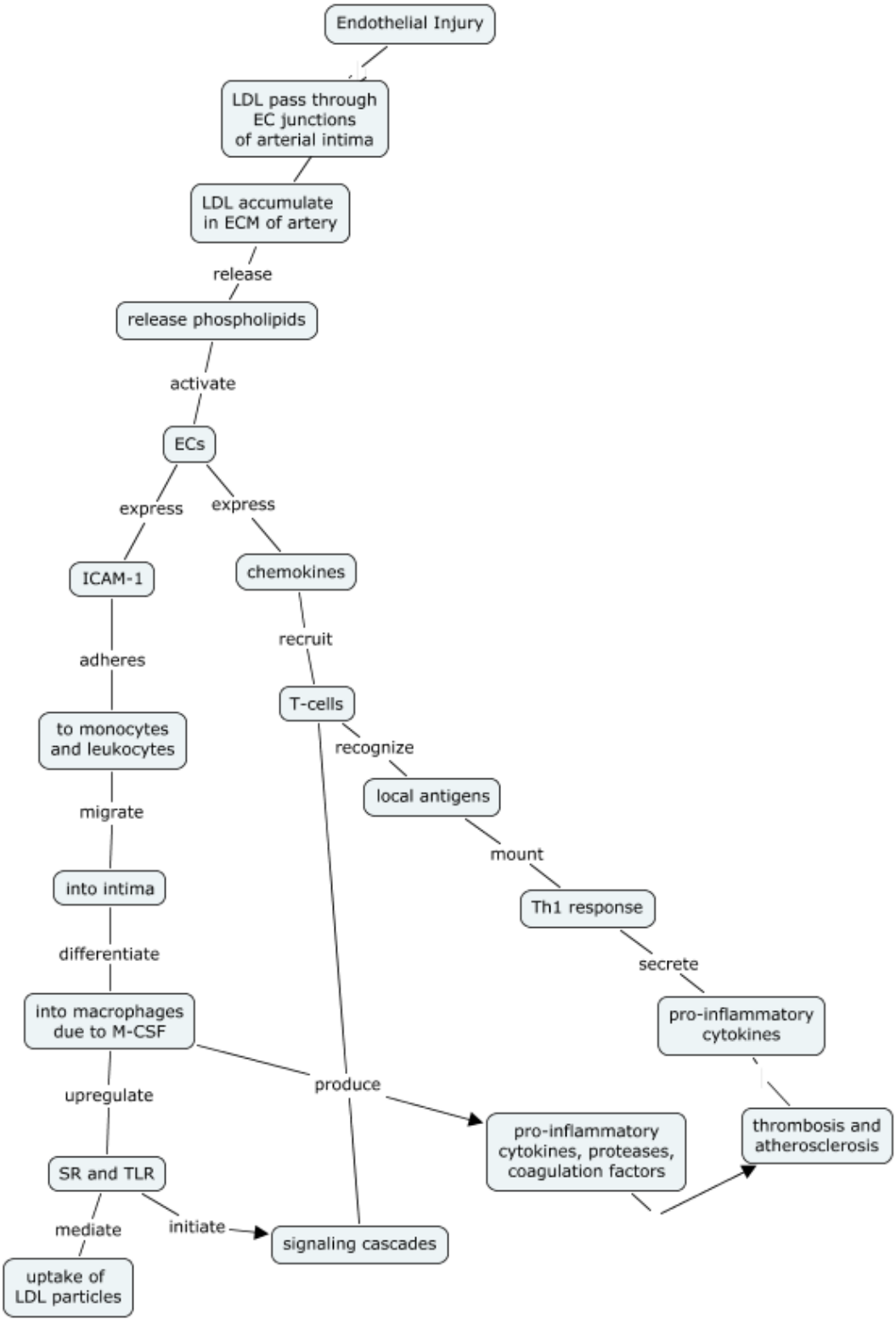

Fig 1.4- Atherosclerosis pathway leading to thrombosis and blockage of blood flow (24).

Inflammation is an important factor contributing to atherosclerosis (51). Local inflammation can lead to plaque instability and ultimately thrombosis. Macrophages release cytokines to recruit leukocytes and increase the presence of ICAM on the cell surface as well as the production of NO. NO leads to vasodilation and an increase in flow and leukocyte delivery, the leukocytes will adhere to adhesion molecules. The circulating monocytes and leukocytes are then brought into the tissue leading to inflammation. Inflammation is also accompanied by decreased bioavailability of NO (51). The inflammation pathway can be seen in Figure 1.5.

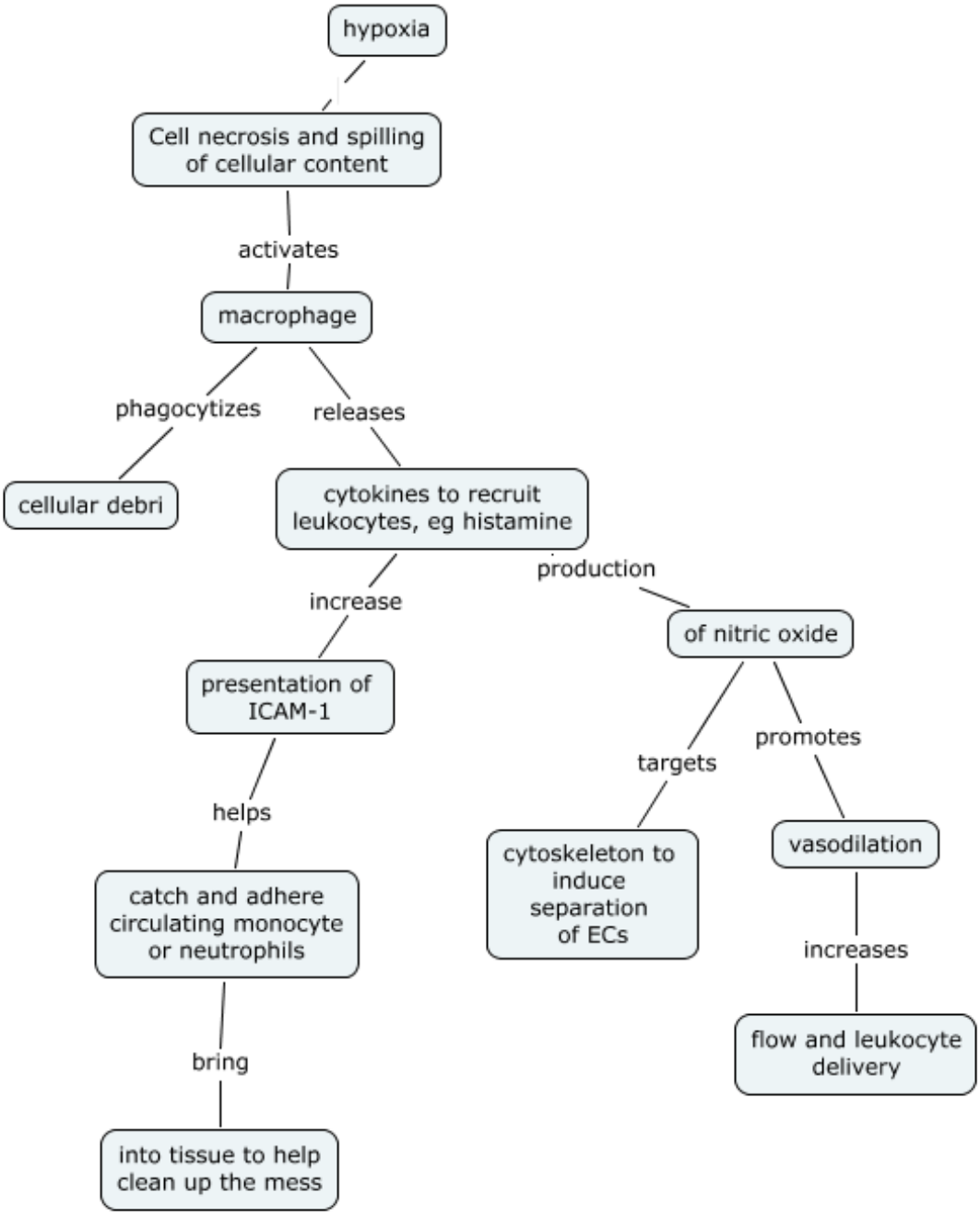

Figure 1.5- Inflammation pathway contributing to atherosclerosis.

Both atherosclerosis and inflammation are present in EC dysfunction and lead to thrombosis and/or the prevention normal blood flow. Associated with these are all of the complications found in diabetes.

Blood Composition

Blood composition is also important to understand in normal and diabetic patients. Normal blood composition consists of low-density lipoprotein (LDL) cholesterol < 100 mg/dL, high-density lipoprotein (HDL) cholesterol > 40 mg/dL and total cholesterol < 200 mg/dL (2, 31). These values can be seen in Table 1. HDL is an important antioxidant and has anti-inflammatory properties (10). HDL plays a role in transporting cholesterol from peripheral cells to the liver to be metabolized and excreted (10). Normal triglyceride levels are considered < 150 mg/dL (31).

Table 1- LDL, HDL and total cholesterol levels as given by the National Institutes of Health.

LDL Cholesterol	
<100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high
160-189	High
≥190	Very high
Total Cholesterol	
<200	Desirable
200-239	Borderline high
≥240	High
HDL Cholesterol	
<40	Low
≥60	High

Diabetic Blood Composition

Research indicates that dyslipidemia and elevated LDL cholesterol is a major cause of cardiovascular disease (10, 31, 41). These are associated with diabetic complications including nephropathy, retinopathy and neuropathy and high triglycerides (2, 31). High LDL is considered to be 160mg/dL, low HDL is considered to be < 40 mg/dL, high total cholesterol is considered to be > 240 mg/dL and high triglycerides are considered to be > 200 mg/dL (2, 31). Once LDL levels are > 160 mg/dL pharmacological therapy is recommended (2).

Dyslipidemia, which is characterized by hypercholesterolemia, hypertriglyceridemia and low HDL, is very prevalent in those with type II diabetes (31). The decrease in HDL levels are related to elevated plasma triglycerides (10). It has also been found that type II diabetic patients who are obese have an even greater decrease in HDL levels than non-obese diabetic patients (10). Type 1 diabetic patients also often have dyslipidemia (31). A study was done that showed type 1 diabetic patients were found to have high total cholesterol and LDL, but HDL and triglycerides were found to be within a normal range (31). Glycemic control has been found to have a strong effect on total cholesterol, LDL and triglyceride concentrations (41).

Stents in Diabetic Population

There are many differences in EC function between healthy patients and diabetic patients so it is not unusual that diabetic patients might react differently to stents. And diabetic patients are often times the people who are in need of stents, as diabetes is associated with cardiovascular disease. 1.5 million revascularization procedures are performed each year in the United States (20). About 25% of patients that undergo

percutaneous coronary intervention have diabetes (52). Diabetic patients have been shown to react differently than non-diabetic patients to implanted stents, often having an increase in restenosis, which is defined as > 50% diameter stenosis (52). Diabetic patients also typically have smaller coronary arteries to begin with, which could be partly to blame for the higher restenosis rate (37). In a study done testing multivessel stenting in diabetic patients it was found that there was a lower one-year survival in diabetic patients that were treated with either oral antigens or insulin compared with non-diabetic patients; about 85% in diabetic patients that were treated with oral antigens vs. 86% in diabetic patients who were treated in insulin, compared to 95% in non-diabetic patients (37). Diabetic patients also had an increase in target lesion revascularization (TLR) (37). Fig. 1.6 shows the survival curve of diabetic patients treated with insulin, diabetic patients treated with an oral agent and non-diabetic patients. An event-free survival was considered to be freedom from death, Q-wave myocardial infarction (QMI) and TLR. Those treated with insulin showed a trend towards an even higher revascularization rate and lower one-year survival rate (37). The decreased performance in diabetic patients may be due to new lesions or untreated previous lesions or a decrease in the revascularization due to diseased coronary arteries (37).

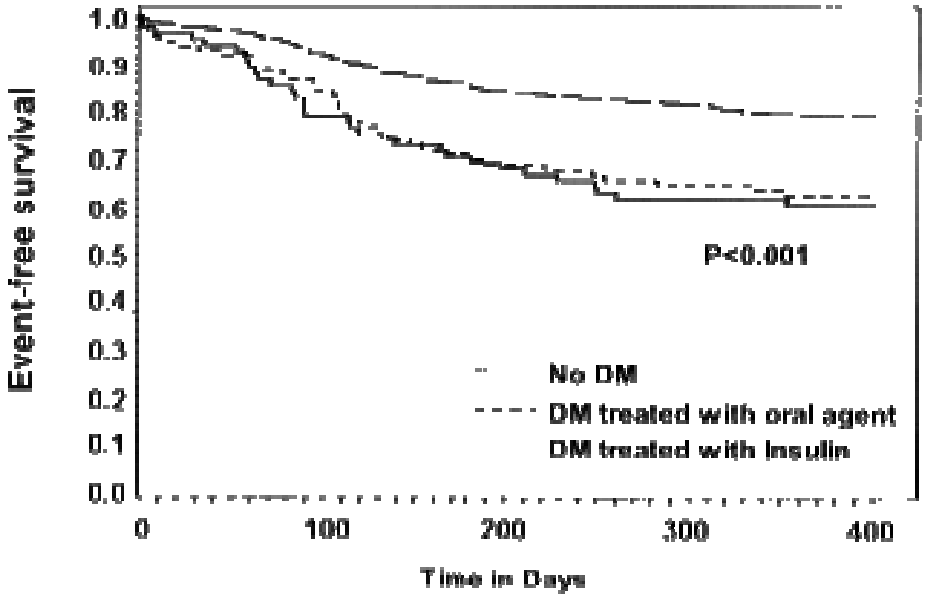

Figure 1.6- Survival curve after multivessel stenting illustrating that diabetic patients have a decreased rate of event-free survival after multivessel stenting (37).

Another study evaluated patients from 16 interventional trials to determine which factors might be most closely related to diabetic restenosis (49). In this study all bare-metal stents were used. Restenosis was found to increase after 6 months in diabetics (31.1%) as compared to non-diabetics (20.6%, P< 0.001). The predictors of restenosis were determined to be vessel reference diameter, stented length of vessel and reduced BMI (49). The main determinant was found to be vessel reference diameter with an increase of 6%, 9% and 13% above non-diabetic restinosis rates for large-, medium-, and small-sized vessels, respectively (49).

The SYNTAX (SYNergy Between PCI With TAXus and Cardia Surgery) study compared coronary artery bypass graft surgery (CABG) and the TAXUS Express paclitaxel-eluting stent (PES) in non-diabetic and diabetic patients with complex left main and/or 3-vessel disease (9). Revascularization in the PES group was lower among

the diabetic patients (49.1%) as compared to the non-diabetic patients (59.3%, P = 0.007), however, no statistical significance was found between the revascularization rate of diabetic and non-diabetic patients treated with CABG (9). Major adverse cardiac and cerebrovascular events (MACCE) was defined as including death, cerebrovascular accident (CVA) and repeat revascularization. In one-year clinical results, MACCE was significantly higher in diabetic patients after PES treatment compared with non-diabetic patients (9). In patients who had medically treated diabetes, PES treatment was a predictor for repeat revascularization but not death, CVS or MI (9). The differences in diabetic patients may be due to inflammation, atherosclerosis or lesion complexity (9).

Another study looked to compare two different types of drug-eluting stents (19) in diabetic patients, paclitaxel-eluting stents (PES) and sirolimus-eluting stents (37, 50). It was found that thrombosis rates were higher in both stents among insulin-treated diabetic patients compared to non-insulin treated patients (PES = 2.1% vs. SES = 2.8%, P = 0.74) (50). The insulin-treated diabetic patients had higher rates of stent thrombosis compared with the non-insulin treated patients (2.5% vs. 0.6%, P = 0.005) (50). Although, they did not find a statistical difference between the two different types of stents, they did find that insulin-treated diabetic patients are at a higher risk for thrombosis.

In patients with diabetes the prevalence of left main artery lesions, multivessel disease and CAD are increased (20). Many studies support the finding that diabetic patients are more likely to suffer restenosis, death or thrombotic complications compared to non-diabetic patients (9, 20, 26, 37, 49, 52). There are several physiological reasons that diabetic patients may fare worse. Platelets are more likely to aggregate and there can be the development of hypertension, EC dysfunction, accelerated atherogenesis and thrombosis (20, 26). Often times diabetic patients are found to have a smaller vessel

diameter and larger vessel occlusion (20). Normally, bypass channels can form to pass an obstruction, however, in diabetic patients there is an impaired ability to form coronary collaterals (20). Diabetic patients are also less likely to undergo remodeling of their coronary arteries to help compensate for an atherosclerotic build up and maintain proper blood flow (20). Diabetes has a great effect on the success of a stent; the creation of a diabetic environment to test a stent would be beneficial to delve into what characteristics might reduce the rates of restenosis and thrombosis in diabetic patients.

Diabetic Models

Due to the somewhat distinct physiology of a subject with diabetes, diabetic models are used to test stents before they proceed into a patient population. Diabetic models help obtain important data to characterize which stents have potential in the clinic. Diabetic animal models are necessary before stents can ultimately make it to the clinic, but the more data that can be obtained from physiologic bench top tests the better. There are currently many diabetic animal models, however, there are also additional challenges and costs associated with using animal models as opposed to further benchtop testing.

Three commonly used mice models are *db/db*, Akita and streptozotocin (STZ) induced C57BL/6J. *db/db* mice are genetically diabetic, they possess a genetic mutation of the leptin receptor and represent a model of type 2 diabetes through the presence of hyperglycemia, obesity, and hyperinsulemia. Heterozygous Akita male mice represent type 1 diabetes through a mutation in the insulin II gene leading to hypoinsulinemia and eventually hyperglycemia (39). Akita mice are very expensive and many of the offspring are not usable; they must be crossed with C57BL/6J mice and homozygous offspring die

in 12 weeks and only heterozygous males can be used (39). STZ and alloxan are mediated by reactive oxygen species; they can both be injected into animals to kill β-cells, therefore rendering them unable to produce insulin, mimicking type 1 diabetes (39). (39). To create C57BL/6J diabetic mice, injections must be given for 5 days and then there is a 4 week latency period at which point some mice still do not portray a diabetic model. This process requires extra time and resources (39).

Non-obese diabetic (NOD) mice are commonly used to study type 1 diabetes. They spontaneously developed and display characteristics of hypercholesteremia and hyperglycemia. In comparisons to humans, however, NOD mice are more often found in females and are more susceptible to other autoimmune diseases. There are also some rats that spontaneously develop type 1 diabetes such as Biobreeding rats, DP-BB rats, Komeda Diabetes Prone rats, LEW.1AR1 rats and LEW.1WR1 rats (35, 50).

Rabbits are also commonly used to study diabetes. A diabetic model can be created with an alloxan injection in New Zealand White rabbits (18). Beagle dogs have also been used to induce diabetes with STZ, this occurs in ten days post injection (7). Diabetic porcine models have been created with STZ in Chinese Guizhou minipigs (52). Rhesus monkey can also be used to study type 1 diabetes through STZ treatment (42). Total pancreatectomy is often used to induce diabetes in animal models (42). In rhesus monkeys, 90% of the pancreas must be removed to cause diabetes leading to much greater complexity and a 7-10 day recovery period (42). This surgery requires about 2-3 hours and requires a professional anesthesiologist to monitor the animal (42). The postoperative procedures are very similar to that of a human (42). The use of a partial pancreatectomy and STZ together lead to a diabetic model after six months in rhesus monkeys (42).

Much time and effort goes in to creating animal models that can mimic diseases. While this is an imperative step, as animal testing creates a more physiologic environment than benchtop tests, more testing can be done to improve data and find the best candidate to pursue further testing. The use of animals for preclinical testing requires more time, money and resources; more expertise and training is needed to properly care for the animals as well as perform surgeries. All animal care must follow proper protocol and procedures in accordance with specific guidelines. The use of a "diabetic" BVM could potentially help to enhance this process by being used as a preclinical test before stents are put into animal models to find the most promising stents. With a "diabetic" environment the affect of diabetes on stents can be looked at more closely and a stent can be designed with a higher success rate in diabetic patients.

Summary and Goals of this Project

The BVM has been created as an *in vitro* testing environment. It is beneficial to get preliminary data regarding stent performance before animal testing is done. The current BVM is designed to be as physiological as possible, although at best it represents a "healthy" vessel environment. With the population of diabetics who end up with cardiovascular disease and due to their varying blood composition as well as the fact that they do not react the same to stents as non-diabetic patients, there is a need for a "diabetic" testing environment to be created.

ECs are normally in a quiescent state; they are imperative for coagulation, leukocyte trafficking and vasomotion. When ECs are activated as in diabetes, the balance is thrown off, the ECs become procoagulant, lose vaso control and this eventually leads to inflammation and atherosclerosis. EC activation has also been found

to be correlated with ICAM-1 expression. ICAM-1 catches circulating monocytes and leukocytes and brings them into the tissue leading to plaque build up. ICAM-1 has been shown to be a marker present in patients with diabetes.

The broader goal of the BVM lab is to create a diabetic environment in the BVM in which to test stents. In order to facilitate this goal, the aim of this project was to create a protocol to test for the presence of ICAM-1, through immunohistochemistry. This protocol will be obtained by testing human tonsil tissue, which is known to express ICAM-1. Once this protocol can positively identify ICAM-1 using tonsil tissue, it will be tested on HUVECs that have been cultured in the BVM on an ePTFE scaffold. Eventually, different stimuli will be added to the media to up-regulate ICAM-1 leading to a "diabetic" environment in which stents can be tested. This will be beneficial for companies to have additional preliminary benchtop tests to perform on a "diabetic" blood vessel mimic. It will save time, money and resources.

CHAPTER 2:

ICAM-1

Introduction

Coronary artery disease is a significant factor in the death of diabetic patients (13). Since diabetic patients react differently to stents than non-diabetic patients, it would be beneficial to have a "diabetic" environment with which to test stents. For the purpose of this research a diabetic environment has been simply defined as an environment in which ICAM-1 is upregulated. The first step in creating a diabetic environment is to be able to test if ICAM-1 is in fact present. To do so a protocol to test for ICAM-1 must be created. Once a working protocol exists, different methods can be investigated to upregulate ICAM-1 and tested using the protocol to see if ICAM-1 is upregulated.

ICAM-1 functions in the movement of leukocytes into tissues (25). ICAM-1 is only synthesized upon activation and participates in leukocyte endothelial cell adhesion in chronic inflammation (25). When there is no inflammation or infection both leukocytes and endothelial cells are quiescent, however, if inflammatory signals are released, ICAM-1 is produced and expressed on the cell surface. ICAM-1 mediates the binding of leukocytes by interacting with carbohydrate ligands on leukocytes. Other secreted activators cause changes in the shape of the leukocytes and activate leukocyte integrins (such as $\alpha L\beta 2$) causing a tighter bond between the leukocytes and ICAM-1. This can be seen in figure 2.1. The leukocytes are then moved into the underlying tissue leading to atherosclerotic plaque build-up and narrowing of the blood vessels (25).

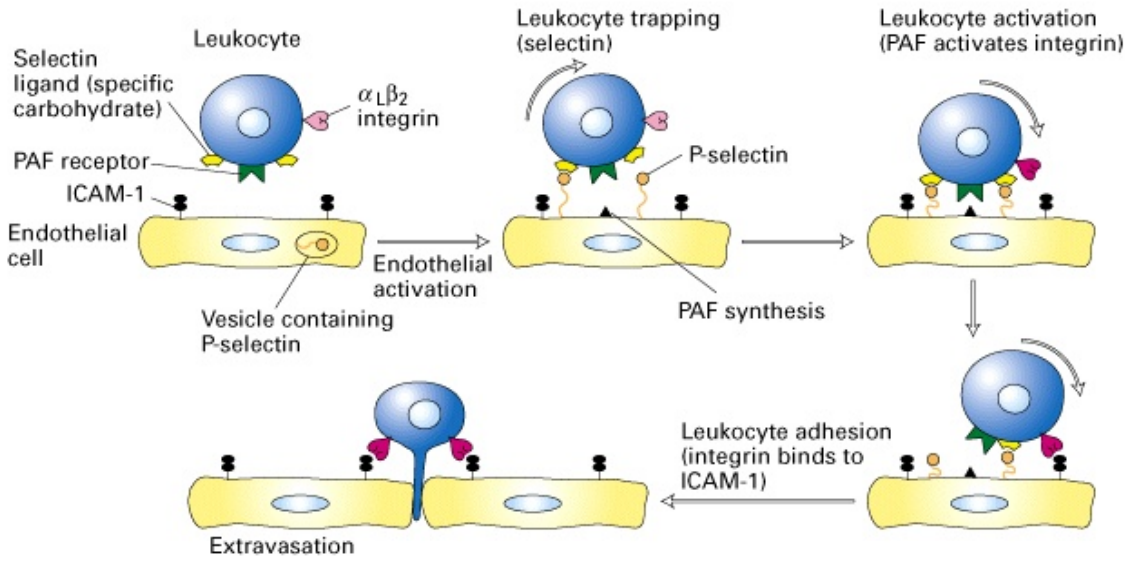

Figure 2.1- ICAM pathway illustrating ICAM-1 expression and leukocyte adhesion (46).

Relevance of ICAM-1

In diabetes, the activation of ECs causes the upregulation of a couple of adhesion molecules. ICAM-1 was chosen to be a marker for a diabetic environment because research showed that ICAM-1 is a powerful predictor of type 2 diabetes. A study done in 2004, looked at the ability of elevated levels of ICAM-1, e-selectin and VCAM-1 to predict the development of type 2 diabetes (38). It was found that e-selectin and ICAM-1 were predictors of type 2 diabetes even when corrected for BMI, family history of diabetes, diet score and postmenopausal hormonal use. Along with these results, it was also found that ICAM-1 was increased ten years prior to the onset of diabetes. It was concluded that elevated plasma levels of ICAM-1 and e-selectin were predictors of type 2 diabetes in initially healthy women (38). Another study analyzed the relationship between insulin resistance and soluble adhesion molecules including ICAM-1, VCAM-1

and e-selectin (45). Soluble adhesion molecules are in the plasma and serve as markers for adhesion molecules. In this research article, it was found that ICAM-1 and e-selectin were inversely correlated with insulin sensitivity (45). This study, however, also found that ICAM-1 was correlated with BMI and total body fat (45).

Yet another study looked at adhesion molecules in obese hypertensive men (19). In this study, ICAM-1, VCAM-1 and e-selectin levels were found to be similar, not related to hypertension, but rather due to obesity. Another study looked at ICAM-1, VCAM-1 and selectins in people who were obese, hypertensive or type 1 diabetic (22). There was an increase of ICAM-1, VCAM-1 and e-selectin in children and adults who were type 1 diabetic, hypertensive or obese (22). In type 1 diabetic children, the levels of ICAM-1 and e-selectin were increased; VCAM-1 and ICAM-1 were dependent upon type 1 diabetic children's triglyceride levels (22). Children who were genetically predisposed to diabetes had an increased level of ICAM-1. There was also an association of ICAM-1 with the risk of myocardial infarction (22). It was concluded that endothelial activation was mostly found to be due to ICAM-1 and e-selectin (22).

ICAM-1 Analysis Techniques

The studies above all support the use of ICAM-1 as a predictor of diabetes, and thus ICAM-1 was chosen as a marker for a diabetic environment in this project. There are a couple of different ways that ICAM-1 can be measured- protein analysis, mRNA analysis, immunohistochemistry and immunfluorescence. All of these techniques could

possibly show if ICAM-1 is present or being upregulated. Certain procedures are better for certain applications.

Protein expression of ICAM-1 can be done using an array biochip (14). This method allows for many different proteins to be analyzed. Different DNA binding sequences or antibodies can be placed onto the biochip to detect proteins in the solution. In one study, researchers took blood samples and kept them at -70°C and then measured for different adhesion molecules in the plasma (14). This is a good method to use when there are cells in a solution that must be analyzed.

Flow cytometry is often used to count populations of interest. It can sort particles based on their properties. Flow cytometry is often used in immunofluorescence. Detectors can pick up the fluctuations in fluorescent emission peaks to determine the amount of fluorescence present (27). In a published study, HUVECs were enzymatically isolated and treated with different concentrations of D-glucose; the effect of D-glucose on inflammation was analyzed. The HUVECs were first trypsinized and then put in a blocking solution followed by a rinse in PBS (8). Next the cells were suspended in the primary antibody and then the primary antibody was detected through the use of a secondary antibody and the flow cytometer can measure the fluorescent intensity (8). The researchers found that increased extracellular D-glucose did not cause ICAM-1 upregulation (8). Another study, that looked at the use of a mushroom to induce ICAM-1, used flow cytometry to analyze the ICAM-1 protein on the cell surface, and polymerase chain reaction (PCR) to analyze the mRNA level of ICAM-1 (29). To measure the protein expression on the cell surface, the cells were stained with anti-human ICAM-1 antibody conjugated with phycoerythrin. The cells were then analyzed with a

flow cytometer and the data displayed the fluorescent intensities. In PCR, part of the DNA will be amplified by going through denaturation and replication cycles; complementary DNA is synthesized to allow for selective replication. mRNA was analyzed to ensure that an increase in mRNA levels of ICAM-1 was correlated to an increase in protein expression levels of ICAM-1.

Immunofluorescence is another very common technique to analyze the expression of ICAM-1 levels. One study looked at the upregulation of ICAM-1 due to lipopolysaccharide (LPS) (43). To use this method, samples were embed in tissue-freezing media and frozen at -70°C. A cryotome was used to cut sections and the samples were then dried at room temperature for two hours. The sections were then treated with a 30% methanol/ 70% acetone mixture (43). The sections were next fixed in paraformaldehyde, washed in PBS (43). They were then incubated in 10% donkey serum in PBS for 30 minutes to block nonspecific binding sites (43). Next was an incubation in the primary antibody at a 1:200 dilution for one hour (43). Then the samples were washed with PBS and incubated in Cy3-labeled donkey anti-rat IgG at a 1:250 dilution for 45 minutes. The samples were then washed with PBS and counterstained with fluorescein-labeled wheat germ agglutinin and bisbenzamide. Finally, the samples were coverslipped (43). This method allowed researchers to see that there was an increase in ICAM-1 expression due to LPS (43). In another study the effect of sustained hypoxia on ICAM-1 was analyzed (12). To do so, rats were exposed to hypobaric hypoxia and then the lung tissue was embedded. The protein level of ICAM-1 was found to increase through immunofluorescent staining. Immunofluorescent staining is a very useful way to determine if a certain protein is present. If the protein is present it will be tagged by the

primary antibody and following reagents leading to the expression of fluorescence, which can easily be seen.

Immunohistochemistry (IHC) is also frequently used to measure ICAM-1 levels. To perform IHC, the samples are fixed, dehydrated and then embeded in paraffin wax (8). Frozen tissue sections and tissue culture cells can also be used as samples (44). If the samples are in paraffin wax, they can be sectioned and then stained with a primary antibody, secondary antibody, AB enzyme reagent, followed by peroxidase substrate (44). One study analyzed the simultaneous expression of ICAM-1, VCAM-1 and vascular endothelial growth factor (VEGF) in diabetic fibrovascular membranes using IHC (28). They used an anti-human ICAM-1 antibody. These samples were also embedded in paraffin wax (28). The first step after sectioning the samples was to rehydrate the samples in alcohols, incubate them in hydrogen peroxide, perform an antigen retrieval step, incubate in a blocking solution, leave them overnight at 4°C with the primary antibody, wash them in PBS, incubate with biotinylated secondary antibody for 60 min, wash in PBS, and finally incubate in streptavidin horseradish peroxidase and chromogen (28). This method allowed researchers to find coexpression of ICAM-1, VCAM-1 and VEGF in most of the fibrovascular membranes they looked at. ICAM-1 was the most common, it was in eight of nine membranes (28). IHC is very similar to immunofluoresence except instead of being tagged with fluorescence, a brown staining will be shown if the protein being tagged is present.

ICAM-1 Expression

The specific aim of this project is to create a protocol to determine if a "diabetic" environment is created in the BVM to test stents by using ICAM-1 as a marker for diabetes. In this project, IHC was the first method used. Tonsil tissue was used as a positive control because research has shown that tonsil expresses adhesion molecules including ICAM-1 (17). A lot of positive staining is typically seen in the high endothelial venules (HEV) in the parafollicular regions, and lower border regions of the reticulated epithelium. ICAM-1 can also be seen on epithelial cells of the crypts (40). The palatine tonsil has openings called crypts that are lined with reticulated epithelium, which contains epithelial cells, lymphocytes and endothelial cells- all of which stained positive for ICAM-1 (30). Figure 2.2 below shows human palatine tonsils stained for ICAM-1; the HEVs of the parafollicular region can be seen as PF, the cryptal epithelium is labeled CE, and the follicular region (F) does not stain positive for ICAM-1 (40). Germinal centers of the human tonsil also have a high uniform expression of ICAM-1 (23). The protocol used in Figure 2.2 consisted of fixing the palatine tonsil in paraformaldehyde and then frozen in liquid nitrogen (40). 30um sections were incubated at 4°C for 48 hours in the primary antibody (anti-ICAM-1). They were the incubated with goat-anti-mouse IgG, silver-enhanced, dehydrated, immersed in propylene oxide, infiltrated with propylene oxide and resin and then embedded in TAAB-resin (40). Figure 2.2 shows what might be expected to stain positive for ICAM-1.

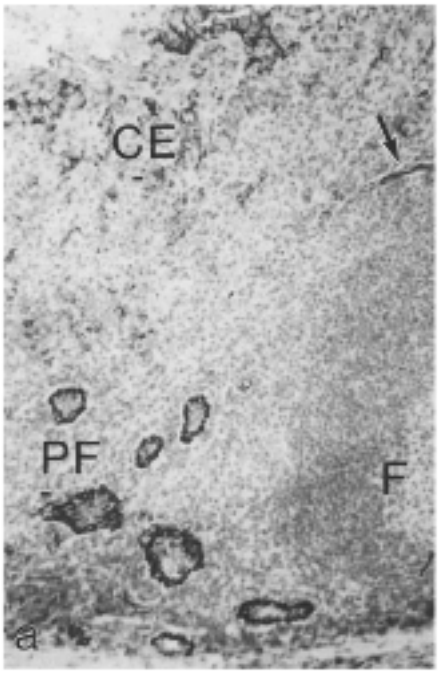

Figure 2.2- Immuno-electron-microscopic staining of ICAM-1 on human palatine tonsils (40).

H&E

Since tonsil tissue is known to express ICAM-1 it was used as a positive control to create an ICAM-1 protocol. Before starting IHC, H&E staining was done to characterize the tissue. Hematoxylin will stain nuclei blue; the eosin stains cytoplasm pink. The first H&E protocol can be seen below. It was verified that this was tonsil tissue by looking at the H&E slides. A microtome was used to make sections of 6um. Pants, closed toed shoes and gloves were worn at all times. All steps were performed in the fume hood, and slides were allowed to dry for 48 hours before viewing in the microscope.

Protocol #1: H&E Staining of human tonsil tissue. October 18, 2009.
Purpose: See the structure and morphology of the tonsil tissue.
Materials: Paraffin embedded tonsil tissue obtained in October, 2009.
Process:

1) 3 minutes in xylene
2) 3 minutes in xylene
3) 3 minutes in xylene
4) 2 minutes in 100% EtOH
5) 2 minutes in 100% EtOH
6) 2 minutes in 95% EtOH
7) 1 minute air dry
8) 4 minutes in hematoxylin
9) 1 minute in distilled H_2O
10) 30-45 seconds in clarifier
11) 1 minute in distilled H_2O
12) I minute in bluing
13) 1 minute in distilled H_2O
14) 1 minute in 95% EtOH
15) 1 minute 30 sconds in eosin
16) 1 minute in 100% EtOH
17) 1 minute in 100% EtOH
18) 1 minute in 100% EtOH
19) 3 minutes in xylene
20) 3 minutes in xylene
21) 3 minutes in xylene

This first protocol created tissue samples that were dark purple so the hematoxylin time was decreased. The next protocol was changed from 4 minutes in hematoxylin to 3 minutes in hematoxylin. This was better, but still a little dark. H&E staining was done one more time with 2:30 minutes in hematoxylin and the samples were much easier to see. The last protocol with hematoxylin time at 2:30 minutes was accepted. Images of the tonsil tissue at all of the different hematoxylin times can be seen below.

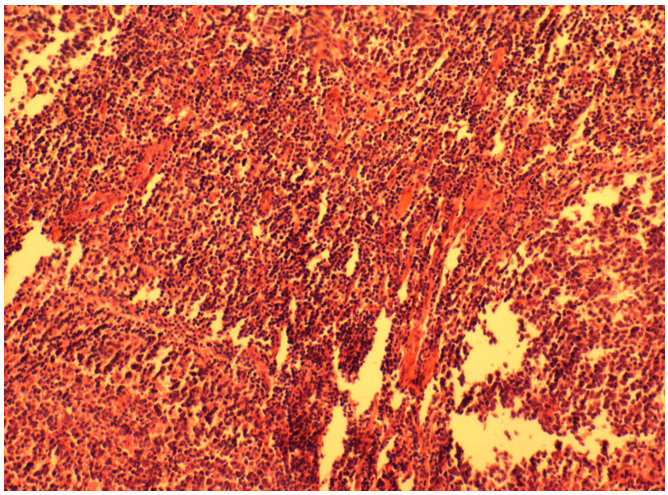

Figure 2.3- Tonsil tissue in hematoxylin for 4 minutes

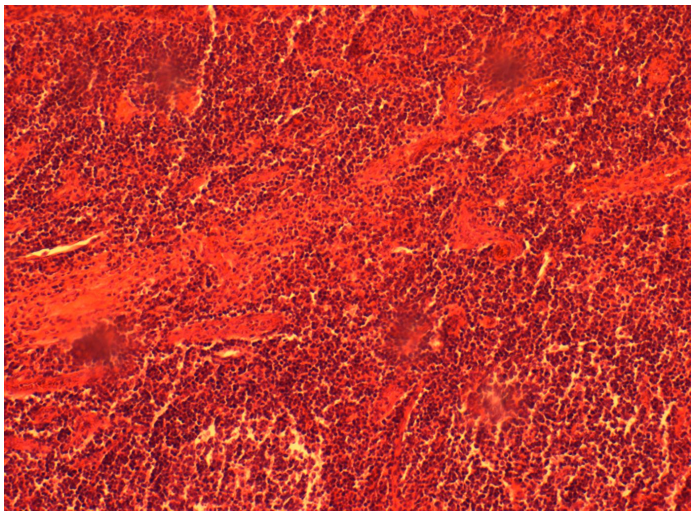

Figure 2.4- Tonsil tissue in hematoxylin for 3 minutes

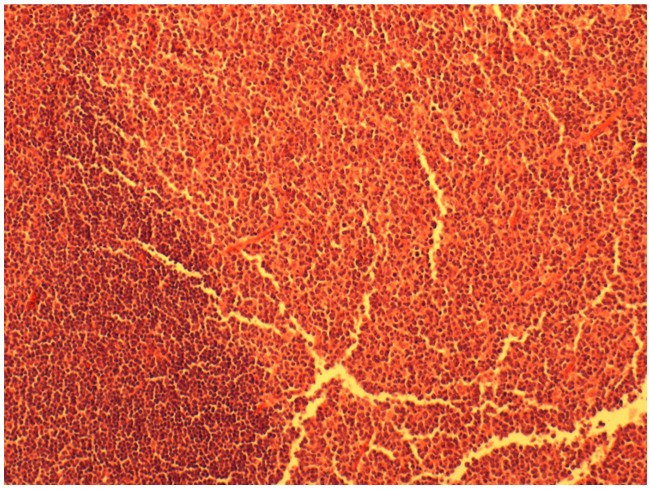

Figure 2.5- Tonsil tissue in hematoxylin for 2:30 minutes

Final Protocol: H&E Staining of human tonsil tissue- Protocol Accepted. January 9, 2010.
Purpose: See the structure and morphology of the tonsil tissue.
Materials: Paraffin embedded tonsil tissue obtained in October, 2009.
Process:

1) 3 minutes in xylene

2) 3 minutes in xylene
3) 3 minutes in xylene
4) 2 minutes in 100% EtOH
5) 2 minutes in 100% EtOH
6) 2 minutes in 95% EtOH
7) 1 minute air dry
8) 2:30 minutes in hematoxylin
9) 1 minute in distilled H_2O
10) 30-45 seconds in clarifier
11) 1 minute in distilled H_2O
12) I minute in bluing
13) 1 minute in distilled H_2O
14) 1 minute in 95% EtOH
15) 1 minute 30 sconds in eosin
16) 1 minute in 100% EtOH
17) 1 minute in 100% EtOH
18) 1 minute in 100% EtOH
19) 3 minutes in xylene
20) 3 minutes in xylene
21) 3 minutes in xylene

Immunohistochemistry

Next an ICAM-1 IHC protocol was worked on. Anti-ICAM-1 antibody, catalog number sc-107 was purchased from Santa Cruz Biotechnology along with mouse ABC system sc-2017. The ABC system comes with suggested instructions ranging in times and concentrations. Protocol #1 is shown below. Before the protocol is begun, the samples are sectioned at 6um. Control slides were incubated in PBS while the experimental slides were incubated with the primary antibody. A Tupperware was used as a humidified chamber, to do so a paper towel was wetted and placed on the bottom of the tupperware. The slides then sat on the paper towel. To aspirate off reagents, one slide was aspirated, the new reagent was put on and then the next slide was aspirated and then a new reagent put on. This continued until all the slides were incubating in the new reagent and prevented the slides from drying out. For safety precautions closed toed

shoes, long pants and gloves should be worn at all times. The AB enzyme reagent should be mixed in the hood. All reagents must be made fresh and if extras are left, disposed of in the waste container.

Protocol #1: Immunohistochemistry analysis of human tonsil tissue using ICAM-1 antibody to create a positive control. October 13, 2009.

Purpose: To stain human tonsil tissue for ICAM for a positive control.

Materials: Paraffin embedded tonsil tissue obtained in October, 2009. ICAM-1 antibody from Santa Cruz Biotechnology. Mouse ABC kit. Control: 09-176-2

Process:

1) Mix PBS as per directions on the packet
   one pouch PBS, pH 7.4 mixed with 1L distilled H_2O
2) *incubate* slides with two changes of PBS for 5 minutes
3) Prepare primary antibody- 1µg/ml

- Pipette 5 µl ICAM antibody
- Pipette 0.995 ml blocking serum

4) *incubate* slides with primary antibody prepared above for 30 minutes
5) Wash with 3 changes of PBS for five minutes each
6) Prepare secondary antibody (green bottle) as follows

- 75µl normal blocking serum
- 5ml PBS
- 25µl secondary antibody stock

7) *incubate* with newly prepared secondary antibody for 30 minutes
8) Prepare antibody enzyme reagent in hood

- 50µl advin
- 50µl biotinylated HRP
- 2.5ml PBS

9) Wash with 3 changes of PBS for 5 minutes each
10) *incubate* sections with antibody enzyme reagent for 30 minutes
11) Wash with three changes of PBS for 5 minutes each
12) Prepare peroxidase substrate

- 1.6ml distilled H_2O
- 5 drops 10x substrate buffer
- 1 drop 50x DAB chromogen
- 1 drop 50x peroxidase substrate

13) *incubate* peroxidase substrate for 5 minutes
14) Wash in distilled water for 5 minutes
15) *incubate* in 95% ethanol twice for ten seconds each

16) *incubate* in 100% ethanol twice for ten seconds each
17) *incubate* in xylene three times for ten seconds each
18) Add 1-2 drops of mounting medium and cover.
19) Let dry for 48 hours

This protocol did not show any positive staining of ICAM-1, endogenous staining were seen in the control and the experimental samples. The next time IHC was attempted, the concentration of ICAM-1 antibody was changed from 1ug/ml to 3ug/ml. This did not show any positive staining either. The ICAM-1 antibody was then increased to 5ug/ml again resulting in no positive staining. The next protocol will be considered protocol #2. This protocol kept the ICAM-1 antibody concentration at 5mg/ml but added dehydration steps at the beginning, added the pap pen and added an hour incubation in normal blocking serum. The pap pen creates a hydrophobic barrier so the solutions will stay over the tissue section; the blocking serum will help to block endogenous staining.

Protocol #2: Immunohistochemistry analysis of human tonsil tissue using ICAM-1 antibody to create a positive control. February 20, 2010.
Purpose: To stain human tonsil tissue for ICAM for a positive control.
Materials: Paraffin embedded tonsil tissue obtained in October, 2009. ICAM-1 antibody from Santa Cruz Biotechnology. Mouse ABC kit. Control: 09-18A, 09-184D
Process:
1) Mix PBS as per directions on the packet
One pouch PBS, pH 7.4 mixed with 1L distilled H_2O
2) *incubate* slides with two changes of PBS for 5 minutes
3) Wash slides in xylene for 5 minutes
4) Wash slides in xylene for 5 minutes
5) Prepare blocking serum

- 75 µl normal blocking serum
- 5 ml PBS
- Remove 0.990 ml to add to ICAM-1 antibody
- Save some to incubate control slide in blocking serum

5) Wash slides in 100% ethanol for 1 minute
6) Wash slides in 100% ethanol for 1 minute
7) Wash slides in 95% ethanol for 1 minute
8) Remove slides, let them dry a little and add pap pen
9) *incubate* slides with blocking serum for 1 hour

- Suction reagent after 1 hour, one slide at a time adding new reagent to each to prevent drying out

10) Prepare primary antibody- 5 μg/ml 30-45 minutes into step 8

- Pipette 25 μl ICAM antibody
- Pipette 0.975 ml blocking serum

11) *incubate* slides with primary antibody prepared above for 30 minutes
12) Wash with 3 changes of PBS for five minutes each
13) Prepare secondary antibody (green bottle) as follows

- 75μl normal blocking serum
- 5ml PBS
- 25μl secondary antibody stock

14) *incubate* with newly prepared secondary antibody for 30 minutes
15) Prepare antibody enzyme reagent in hood

- 50μl advin
- 50μl biotinylated HRP
- 2.5ml PBS

16) Wash with 3 changes of PBS for 5 minutes each
17) *incubate* sections with antibody enzyme reagent for 30 minutes
18) Wash with three changes of PBS for 5 minutes each
19) Prepare peroxidase substrate

- 1.6ml distilled H_2O
- 5 drops 10x substrate buffer
- 1 drop 50x DAB chromogen
- 1 drop 50x peroxidase substrate

20) *incubate* peroxidase substrate for 5 minutes
21) Wash in distilled water for 5 minutes
22) *incubate* in 95% ethanol twice for ten seconds each
23) *incubate* in 100% ethanol twice for ten seconds each
24) *incubate* in xylene three times for ten seconds each
25) Add 1-2 drops of mounting medium and cover.
26) Let dry for 48 hours

This protocol was not successful. The next IHC protocol removed the PBS step before the slides were put into xylene for the first time, and added a hydrogen peroxide step for five minutes after the pap pen was applied to the samples. This was not successful. The next protocol was done with some of the samples in hydrogen peroxide

and some without hydrogen peroxide. When the hydrogen peroxide is used, less endogenous staining is seen in the tonsil tissue, however, there is still endogenous staining in both the samples treated with ICAM-1 antibody and those without. IHC images from this protocol can be seen in Figure 2.6 and 2.7. The tonsil tissue was washed in PBS by putting them in dishes in some cases and pipetting PBS over the samples in other times. Neither of these techniques made a difference. The next time IHC was attempted, the hydrogen peroxide step was omitted and the samples were incubated with the primary antibody overnight at 4°C instead of for 30 minutes at room temperature. The samples were also incubated in the peroxidase substrate for ten minutes, as opposed to five minutes. The controls were left in PBS overnight; still no positive ICAM-1 staining was seen.

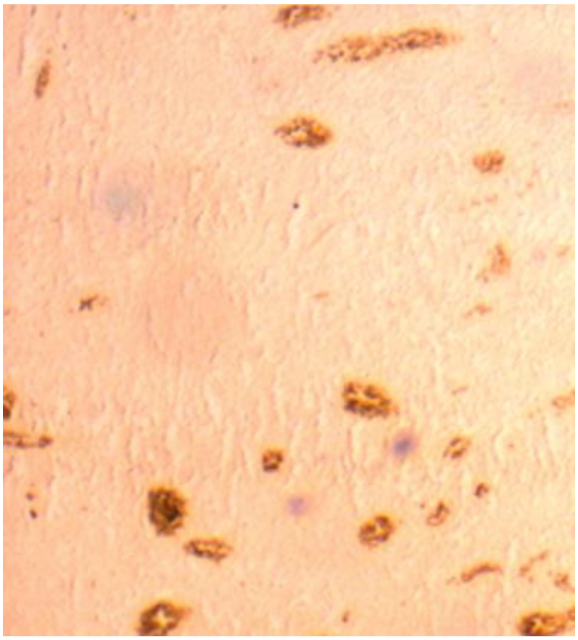

Figure 2.6- Tonsil tissue without hydrogen peroxide

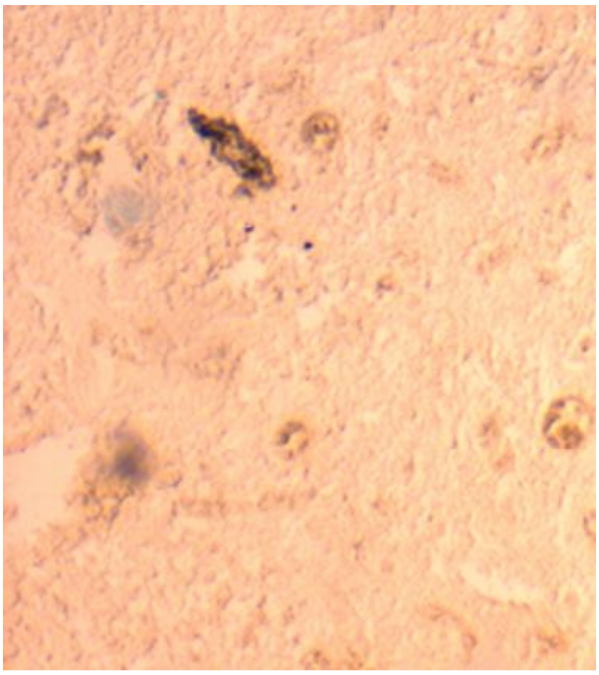

Figure 2.7- Tonsil tissue with hydrogen peroxide

The next trial included an antigen retrieval step, increased the dehydration steps, and did not have a hydrogen peroxide step. Sometimes when samples are embedded in paraffin wax, the wax can block the antigen from binding to the primary antibody; using an antigen retrieval step can unmask the antigen. This heat treatment step was done by incubating the samples in a 0.05% saponin solution. Again, no positive staining was seen; both the control and the experimental slides showed brown staining. The next protocol included the same antigen retrieval step with saponin, but also included a hydrogen peroxide step. Again, no positive staining was found. For the next protocol the hydrogen peroxide was found to have been expired so it was omitted and sodium citrate was used for an antigen retrieval step. Sodium citrate was ordered from Santa Cruz Biotechnology, sc-203383. This protocol will be considered protocol #3.

Protocol #3: Immunohistochemistry analysis of human tonsil tissue using ICAM-1 antibody to create a positive control. April 17, 2010.

Purpose: To stain human tonsil tissue for ICAM for a positive control.
Materials: Paraffin embedded tonsil tissue obtained in October, 2009. ICAM-1 antibody from Santa Cruz Biotechnology. Mouse ABC kit. Control: 09-180B, 09-183B
Process:
1) *incubate* slides in the oven for 10 minutes
2) Let slides cool for 5 minutes
3) Wash slides in xylene for 5 minutes
4) Mix PBS as per directions on the packet
One pouch PBS, pH 7.4 mixed with 1L distilled H_2O
5) Wash slides in xylene for 5 minutes
6) Wash slides in xylene for 5 minutes
7) Wash slides in 100% ethanol for 10 minutes
8) Wash slides in 100% ethanol for 10 minutes
9) Wash slides in 95% ethanol for 10 minutes
10) Wash slides in 95% ethanol for 10 minutes
11) Heat 10mM sodium citrate buffer, pH 6.0

- 95°C

12) *incubate* slides in distilled water for 1 minute with stirring
13) *incubate* slides in 95°C sodium citrate buffer for 5 minutes

- use incubator to keep sodium citrate hot

14) Heat new 10mM sodium citrate buffer for 5 minutes
15) *incubate* slides in 95°C sodium citrate buffer for 5 minutes

- use incubator to keep sodium citrate hot

16) Allow slides to cool in sodium citrate buffer for 20 minutes
17) Wash in distilled H_2O three times for 2 minutes each
18) Add pap pen to samples
19) Prepare blocking serum

- 75 µl normal blocking serum
- 5 ml PBS
- Remove 0.975 ml to add to ICAM-1 antibody
- Save some to incubate control slide in blocking serum

20) *incubate* slides with blocking serum for 1 hour

- Suction reagent after 1 hour, one slide at a time adding new reagent to each to prevent drying out

21) Prepare primary antibody- 5 µg/ml 30-45 minutes into step 8

- Pipette 25 µl ICAM-1 antibody
- Pipette 0.975 ml blocking serum

22) *incubate* slides with primary antibody prepared above for 30 minutes at room temperature
23) Wash with 3 changes of PBS for five minutes each
24) Prepare secondary antibody (green bottle) as follows

- 75µl normal blocking serum
- 5ml PBS
- 25µl secondary antibody stock

25) *incubate* with newly prepared secondary antibody for 30 minutes
26) Prepare antibody enzyme reagent in hood- must sit for 30 minutes

- 50µl advin
- 50µl biotinylated HRP
- 2.5ml PBS

27) Wash with 3 changes of PBS for 5 minutes each
28) *incubate* sections with antibody enzyme reagent for 30 minutes
29) Wash with three changes of PBS for 5 minutes each
30) Prepare peroxidase substrate

- 1.6ml distilled H_2O
- 5 drops 10x substrate buffer
- 1 drop 50x DAB chromogen
- 1 drop 50x peroxidase substrate

31) *incubate* peroxidase substrate for 5 minutes
32) Wash in distilled water for 5 minutes
33) *incubate* in 95% ethanol twice for ten seconds each
34) *incubate* in 100% ethanol twice for ten seconds each
35) *incubate* in xylene three times for ten seconds each
36) Add 1-2 drops of mounting medium and cover
37) Let dry for 48 hours

In this protocol, the sodium citrate was heated in the microwave and then put into the incubator to keep warm. However, the incubator only kept the sodium citrate at 80°C, which is much lower than the recommended 95°C. In the next try, sodium citrate was used (still did not reach the desired temperature) and the blocking serum incubation for one hour was omitted. In the next protocol the sodium citrate was heated in a beaker (from VWR, which has a heat rating of 400°C) on a hot plate and the solution was kept at

95°C. The hydrogen peroxide was also used. It was later found that the blocking serum used in protocol #3 and the following protocols was mouse serum and should have been goat serum. None of these protocols showed any positive ICAM-1 staining, there was a lot of endogenous staining seen on all of the slides.

Immunofluorescence

Due to the high amount of endogenous staining seen, it was decided to switch to immunofluorescence. Ultra cruz mounting medium (sc-24941), goat anti-mouse IgG-FITC (sc-2010), and goat blocking serum (sc-2043) were ordered from Santa Cruz Biotechnology. The initial immunofluorescence protocol will be considered protocol #1 and can be seen below. Tonsil tissue was still used for the positive control.

Protocol #1: Immunofluorescent analysis of human tonsil tissue using anti-ICAM-1 antibody to create a positive control. May 9, 2010.
Purpose: To stain human tonsil tissue for ICAM for a positive control.
Materials: Paraffin embedded tonsil tissue obtained in October, 2009. ICAM-1 antibody from Santa Cruz Biotechnology. Goat anti-mouse IgG-FITC. Goat blocking serum. Control: 09-180C, 09-184C.
Process:
1) *incubate* slides in the oven for 10 minutes
2) Let slides cool for 5 minutes
3) Wash slides in xylene for 5 minutes
4) Mix PBS as per directions on the packet

- One pouch PBS, pH 7.4 mixed with 1L distilled H_2O

5) Wash slides in xylene for 5 minutes
6) Wash slides in xylene for 5 minutes
7) Wash slides in 100% ethanol for 10 minutes
8) Heat 10mM sodium citrate buffer, pH 6.0 in a 1 L VWR beaker on a hot plate until it boils

- 95°C

9) Wash slides in 100% ethanol for 10 minutes

10) Wash slides in 95% ethanol for 10 minutes
11) Wash slides in 95% ethanol for 10 minutes
12) *incubate* slides in distilled water for 1 minute with stirring
13) *incubate* slides in 95°C sodium citrate buffer for 5 minutes

- use hot plate to keep sodium citrate hot
- put slide rack in 1 L VWR beaker

14) Top 10mM sodium citrate buffer to 500 ml in 1 L VWR beaker
15) *incubate* slides in 95°C sodium citrate buffer for 5 minutes

- use hot plate to keep sodium citrate hot

16) Allow slides to cool in sodium citrate buffer for 20 minutes
17) Wash in distilled H_2O three times for 2 minutes each
18) Prepare 10% normal blocking serum- goat

- 0.25 ml normal blocking serum (goat)
- 2.25 ml PBS
- This can be replaced with bovine serum albumin

19) Add pap pen to samples
20) *incubate* in normal blocking serum for 20 minutes
21) Wash with PBS- 5 minutes
22) Prepare primary antibody

- 15 µl normal blocking serum
- 0.985 ml PBS
- 20 µl anti-ICAM antibody → 1:50 dilution
- can try 0.5- 5.0µg/ml

23) *incubate* slides with primary antibody for 1 hour

- Suction reagent after 1 hour, one slide at a time adding new reagant to each to prevent drying out

24) Wash with 3 changes of PBS for five minutes each
25) Prepare fluorochrome-conjugated secondary antibody

- 30 µl normal blocking serum
- 1.97 ml PBS
- 15 µl secondary antibody
- Can try 1.0- 5.0µg/ml (this one is 3µg/ml)
- Can mix normal blocking serum to be 1.5% (now) to 3%

26) *incubate* with newly prepared secondary antibody for 45 minutes

- Must be in a dark chamber

27) Wash with 3 changes of PBS for 5 minutes each

28) Mount coverslip with aqueous mounting medium or 90% glycerol in PBS

After protocol #1, background staining was seen in the control and experimental slides. Images from protocol #1 can be seen in figure 2.8 and 2.9. In the next protocol, the FITC concentration was adjusted from a 3mg/ml dilution to a 1ug/ml dilution (1:500). It was also found that some of the beakers do not fit the slide racks- even beakers with the same catalog number. From then on the beaker was tested with the slide rack to ensure the slide rack will fit into the beaker before sodium citrate was added to the beaker and heated. The next protocol diluted the FITC even more to a 1:1000 dilution. Still background staining was seen, as shown in Figures 2.10 and 2.11. There is nothing known in tonsil tissue that the secondary tissue should be binding too. Both tonsil tissue and the reagents from Santa Cruz Biotechnology have been cited in research articles. To test for background staining both a different species secondary antibody was tried and culturing HUVECs as opposed to using tonsil tissue was tried. This resulted in background staining still being seen. Since the background staining is seen with both secondary antibodies, it is likely that the background staining could be due to the sample rather than the reagents.

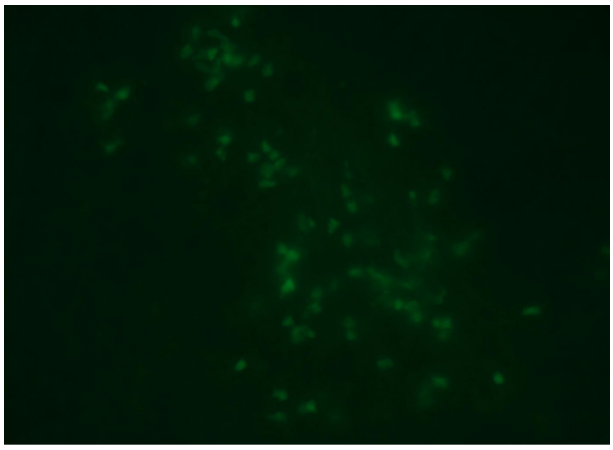

Figure 2.8- Immunofluorescent image of control (w/o primary antibody)

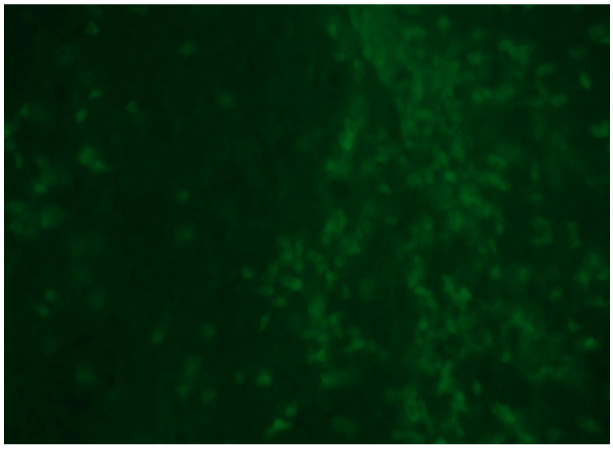

Figure 2.9- Immunofluorescent image of experimental tissue, protocol #1

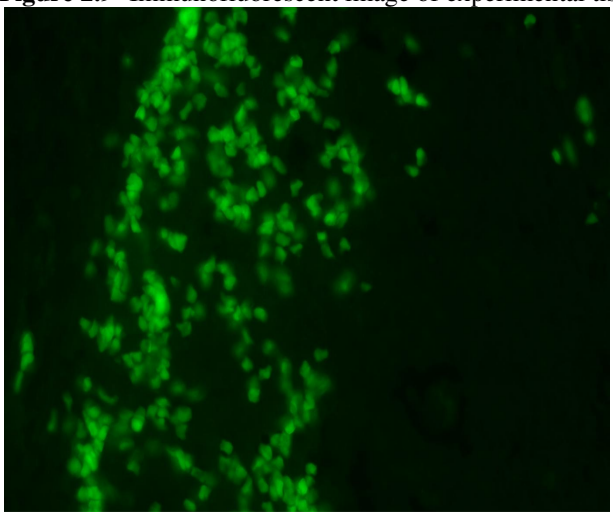

Figure 2.10- Immunofluorescent image of control (w/o primary antibody), 1:1000 FITC dilution

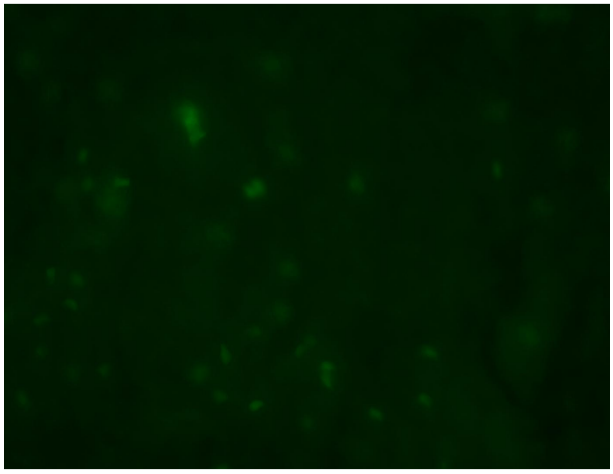

Figure 2.11- Immunofluorescent image of experimental tissue, 1:1000 FITC dilution

The immunofluorescent protocol on HUVECs can be seen below.

Protocol #2: Immunofluorescent analysis of HUVECS using anti-ICAM-1 antibody to create a positive control. May 20, 2010.
Purpose: To stain human tonsil tissue for ICAM for a positive control.
Materials: HUVECs p9. ICAM-1 antibody from Santa Cruz Biotechnology. Goat anti-mouse IgG-FITC. Goat blocking serum. Dextran. Control: cultured in DMEM, not dextran
Process:
1) Make dextran: 10%, 20%, 30% (and DMEM = control)

- 1ml = 1 gram
- 0.63 grams dextran: 9.37 ml DMEDM
- 1.4 grams dextran: 8.6 ml DMEM
- 1.75 grams dextran: 8.25 ml DMEM

2) Culture cells with dextran for 24 hours
3) Fix cells: 5 minutes in -10º C methanol, air dry
4) Wash cells in three changes of PBS
5) Mix PBS as per directions on the packet

- One pouch PBS, pH 7.4 mixed with 1L distilled H_2O

6) Prepare 10% normal blocking serum- goat

- 0.20 ml normal blocking serum (goat)
- 1.8 ml PBS
- This can be replaced with bovine serum albumin

7) *Incubate* in normal blocking serum for 20 minutes
8) Wash with PBS- 5 minutes
9) Prepare primary antibody

- 15 µl normal blocking serum
- 0.985 ml PBS
- 20 µl anti-ICAM antibody → 1:50 dilution
- can try 0.5- 5.0µg/ml

10) *Incubate* slides with primary antibody for 1 hour

- Suction reagant after 1 hour, one slide at a time adding new reagant to each to prevent drying out

11) Wash with 3 changes of PBS for five minutes each
12) Prepare fluorochrome-conjugated secondary antibody (recommend 1:500 dilution) this is 1:1000 dilution

- 60 µl normal blocking serum
- 1.94 ml PBS
- 2 µl secondary antibody
- Can try 1.0- 5.0µg/ml
- Can mix normal blocking serum to be 1.5% to 3% (now)

13) *Incubate* with newly prepared secondary antibody for 45 minutes

- Must be in a dark chamber

14) Wash with 3 changes of PBS for 5 minutes each
15) Mount coverslip with aqueous mounting medium or 90% glycerol in PBS.

When the HUVECs were imaged, it was found that there were no cells attached to the four well plates. The most likely reason is due to the cells not properly being fixed. The temperature of the methanol was not measured before it was put onto the cells, however, the methanol was left in the freezer for 24 hours before being used. The methanol must also be allowed some time to air dry without drying the cells out- this could have needed to air dry for longer.

The immunofluorescent protocol used with the donkey anti-mouse IgG-FITC can be seen below. There were some changes in this protocol compared to protocol #2. The PBS wash after the *incubation* with the normal blocking serum was changed from 5 minutes to one minute. The control slides were incubated with a 10% normal blocking serum in PBS instead of just PBS. After the *incubation* with FITC there were three PBS washes added, then three five minute incubations and then three rinses before being coverslipped.

Protocol #3: Immunofluorescent analysis of human tonsil tissue using anti-ICAM-1 antibody to create a positive control. May 22, 2010.
Purpose: To stain human tonsil tissue for ICAM-1 for a positive control.

Materials: Paraffin embedded tonsil tissue obtained in October, 2009. HUVECS on ePTFE embed in paraffin wax. ICAM-1 antibody from Santa Cruz Biotechnology. Donkey anti-mouse IgG-FITC. Donkey blocking serum. Control: 09-178C

Process:

1) *Incubate* slides in the oven for 10 minutes
2) Let slides cool for 5 minutes
3) Wash slides in xylene for 5 minutes
4) Mix PBS as per directions on the packet

- One pouch PBS, pH 7.4 mixed with 1L distilled H_2O

5) Wash slides in xylene for 5 minutes
6) Wash slides in xylene for 5 minutes
7) Wash slides in 100% ethanol for 10 minutes
8) Heat 10mM sodium citrate buffer, pH 6.0 in a 1 L VWR beaker on a hot plate until it boils

- 95°C

9) Wash slides in 100% ethanol for 10 minutes
10) Wash slides in 95% ethanol for 10 minutes
11) Wash slides in 95% ethanol for 10 minutes
12) *Incubate* slides in distilled water for 1 minute with stirring
13) *Incubate* slides in 95°C sodium citrate buffer for 5 minutes

- use hot plate to keep sodium citrate hot
- put slide rack in 1 L VWR beaker

14) Top 10mM sodium citrate buffer to 500 ml in 1 L VWR beaker
15) *Incubate* slides in 95°C sodium citrate buffer for 5 minutes

- use hot plate to keep sodium citrate hot

16) Allow slides to cool in sodium citrate buffer for 20 minutes
17) Wash in distilled H_2O three times for 2 minutes each
18) Prepare 10% normal blocking serum- donkey

- 0.20 ml normal blocking serum (donkey)
- 1.8 ml PBS
- This can be replaced with bovine serum albumin

19) Add pap pen to samples
20) *Incubate* in normal blocking serum for 20 minutes
21) Wash with PBS- 5 minutes
22) Prepare primary antibody

- 15 µl normal blocking serum
- 0.985 ml PBS
- 20 µl anit-ICAM-1 antibody → 1:50 dilution

- can try 0.5- 5.0µg/ml

23) *Incubate* slides with primary antibody for 1 hour

- Suction reagant after 1 hour, one slide at a time adding new reagant to each to prevent drying out

24) Wash with 3 changes of PBS for five minutes each
25) Prepare fluorochrome-conjugated secondary antibody (recommend 1:500 dilution) this is 1:1000 dilution

- 60 µl normal blocking serum
- 1.94 ml PBS
- 2 µl secondary antibody
- Can try 1.0- 5.0µg/ml
- Can mix normal blocking serum to be 1.5% to 3% (now)

26) *Incubate* with newly prepared secondary antibody for 45 minutes

- Must be in a dark chamber

27) Wash with 3 changes of PBS for 5 minutes each
28) Mount coverslip with aqueous mounting medium or 90% glycerol in PBS

During this protocol, the HUVECs on ePTFE all fell off of the slide during the heat treatment step. To retry the HUVECs without the heat treatment step, protocol #3 was adhered to with the omittance of the heat treatment step. The tonsil tissue in protocol#3 still showed background staining. It was found that the hydrophobic barrier pen that is used to keep the reagents over the sample fluoresces. Vector Laboratories was contacted and it was stated that the pen and its residue does fluoresce, however, this should not affect the tissue samples. Images of this can be seen in figure 2.12. The fluorescing of the tonsil tissue does not look like residue of the pen; it looks like circular cells and the cells are fixed before they come into contact with the pen so their expression will not be altered.

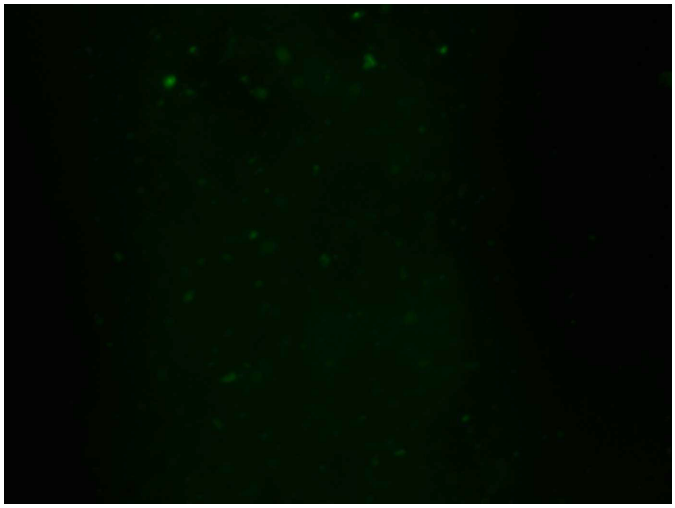

Figure 2.12- Fluorescence of the hydrophobic barrier pen.

Summary

After protocol #3, due to the continuing result of non-specific staining, the tonsil tissue was further analyzed to try to figure out why there is background staining. Even using both IHC and immunofluorescence, background staining was seen. Concentrations of reagents were changed, incubation times were changed, and secondary antibodies were changed. None of these attempts lead to successful identification of ICAM-1 on the tonsil tissue.

The tonsil tissue, received from Central Coast Pathology was most likely fixed in formalin. The time that the samples are in formalin can greatly increase the background staining. Since the tonsil tissue was received already fixed, it is unknown how long they were in formalin. Therefore, further methods need to be analyzed to create a positive ICAM-1 protocol using tissue that can be fixed on the Cal Poly campus to ensure proper fixation for use with IHC and immunofluorescence.

On the Santa Cruz Biotechnology website, research articles are listed that use the same ICAM-1 antibody. Dr. Hsinyu Lee was contacted about the article

“Lysophospholipids increase ICAM-1 expression in HUVEC through a Gi- and NF-{kappa}B-dependent mechanism Lee” (34) in which immunofluorescence was used. Dr. Anil Kumar Bhunia was also contacted about his research article "Lactosylceramide Mediates Tumor Necrosis Factor-induced Intercellular Adhesion Molecule-1 (ICAM-1) Expression and the Adhesion of Neutrophil in Human Umbilical Vein Endothelial Cells,” in this article immunofluorescence and IHC was used.

CHAPTER 3:
SUMMARY AND CONCLUDING REMARKS

Summary

This project worked towards the goal of creating an ICAM-1 protocol to be used in creating a "diabetic" environment. In summary, a "diabetic" environment in which to test stents would be very beneficial. Diabetes is marked by endothelial dysfunction leading to the upregulation of ICAM-1, procoagulation, inflammation and decreased vasodilation. ICAM-1 causes leukocytes to migrate into the vessel leading to an atherosclerotic plaque build up, which can rupture and form a clot, or impede blood flow. It is widely accepted that diabetic patients react differently to stents than non-diabetic patients; this is thought to be due to inflammation, lesion complexity and atherosclerosis, all commonly found in diabetic patients (9). With 6% of the population in the US having diabetes, and a high rate of cardiovascular disease in diabetics, a better treatment is imperative (16). ICAM-1 is an appropriate marker for diabetes and can be used to measure if a "diabetic" environment has been created in the BVM. Studies have shown that elevated plasma levels of ICAM-1 and e-selectin are predictors of diabetes (38). Once a protocol is created, different methods of ICAM-1 upregulation can be tested in the BVM to ultimately lead to the end goal of testing stents in the BVM. If ICAM-1 can be upregulated in the BVM and then stents are tested it would be time and cost effective to be able to characterization the stents *in vitro* before animal models are used.

Challenges

H&E, immunohistochemistry and immunofluorescence were investigated and performed as part of this project. Background staining was seen through both IHC and immunofluorescence; further investigation is required to create a working ICAM-1

protocol. The tonsil tissue seemed very sensitive to the hematoxylin and the time in hematoxylin had to be reduced to 2:30 minutes.

Throughout the attempts of IHC, brown staining was seen in the experimental and control samples. When hydrogen peroxide was used the expression did not seem to be as dark. The changing of the time in the peroxidase substrate did not make much of a difference and the change in the concentration of anti-ICAM-1 antibody did not have much of an affect either. The addition of heat treatment to unmask the antigen also did not change the background staining seen.

When the protocol was switched to immunofluorescence, the same problem persisted. Green fluorescing was seen throughout much of the tonsil tissue. Different concentrations were attempted. Even at a FITC dilution of 1:1000, fluorescing was still seen. To attempt to fix this problem, a different secondary antibody was used and there was still background staining. HUVECs were cultured and used as well, however the fixation process must be perfected. This process could hold potential, however, the process of upregulating ICAM-1 is experimental as well. If there is an error in the process of upregulating ICAM-1 then ICAM-1 would not be expressed. If ICAM-1 is not expressed, it would not be known, so therefore it would be unknown if the error were in the protocol of upregulating ICAM-1 or detecting if ICAM-1 is upregulated. The HUVECs on ePTFE did show a little bit of fluorescing, however, more so when the hydrophobic barrier pen was used, which was later found to be fluorescent itself.

Future Work

Future studies could find an alternative to the hydrophobic barrier pen, such examples include wax from a candle or a crayon. These samples could then be compared to the tonsil tissue where the hydrophobic barrier pen was used. Another option would be to try the HUVECs on ePTFE without the hydrophobic barrier pen, however, this would still require that a positive ICAM-1 source be found. This would be useful to see if the background staining is only related to the tonsil tissue.

A different source of samples that are positive for ICAM-1 must also be found. It is likely due to the fixation of the tonsil tissue that is causing the background staining. Since the tonsil tissue cannot be obtained and fixed on the Cal Poly campus, another source must be found. It may be possible to find what tissue in mice would express ICAM-1. This tissue could then be obtained and fixed in histochoice as opposed to formalin to prevent the background staining.

Cultured cells can also be analyzed further to get a working protocol, if they are fixed properly they should remain in the well plates. If dextran is used however, it is uncertain if the protocol of adding dextran, the percent dilution, and incubation time, will upregulate ICAM-1. So if the protocol is used and no ICAM-1 is seen, it will be unknown whether it is due to an incorrect protocol or if the HUVECs are not expressing ICAM-1. Santa Cruz Biotechnology uses Hela cells as a positive control for ICAM-1, however these cells have been known to be able to take over other cell cultures and could be risky to use in the hood with other cells.

Also, more researchers who have used the same antibody from Santa Cruz Biotechnology can be contacted to see if they can provide their working protocol, which could be mimicked.

All of these are issues that can be assessed further to create a working protocol of ICAM-1 leading to the eventual goal of finding stents with the most potential in treating diabetic patients.

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